RESPONSES OF CELLS TO
pH CHANGES IN THE MEDIUM

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ABSTRACT

Studies were made with time-lapse motion pictures of the reactions of cells in culture to changes in their environment. The concentrations of H^+, HCO_3^- and CO_2 in the medium were altered in such a way that each, in turn, could be maintained constant while the others were varied. Observations were made on the shape of the cells, their activity, and their relation to the substratum. Characteristic reversible changes in the cells were observed whenever environmental pH was altered. Elevation of the pH accelerated cell movements and caused contraction of the cytoplasm, while lowering of the pH retarded and eventually stopped all cell activity, causing apparent gelation of the protoplasm. These responses did not occur when HCO_3^- and CO_2 were varied without changing the pH. It is suggested that local pH changes in the micro-environment of a cell's surface may be a significant factor in controlling cell behavior in culture and *in vivo*.

Many studies have been conducted to determine the chemical and physical conditions required to maintain cells in active proliferation and growth. Relatively little, however, has been done to investigate conditions in the environment of cells which may more immediately determine or alter their activities. Some of the earlier observations on the response of plant and animal cells to changes in hydrogen ion concentration and partial pressures of atmospheric gases have been reviewed by Heilbrunn (7) and by Brooks and Brooks (2).

Movements in the more active cells, such as amebae, have been found to be closely related to viscosity changes in their cytoplasm. This was first interpreted in terms of tensions caused by contraction of the cytoplasm which supposedly accompanied its sol-gel transformation, or which occurred in syneresis of the gel (9, 12). More recently, however, the interpretation of cytoplasmic movement has been re-examined in the light of possible changes in configuration of long-chain protein molecules and the shifting of chemical bonds between them (6, 11, 18).

Important relationships have been shown to exist between the activities of cells and the surface structure of solids with which they come into contact (15, 19). The molecular characteristics of these surfaces can be modified by the complex chemical constituents of the liquid in the cell environment, including the products of the cells themselves (14, 20), and these, in turn, determine the affinity between the cell membrane and the surface and affect the activity and morphology which the cells will assume (19, 24).

Thus, the movements within the cytoplasm of cells as well as the physical relationships of cells to each other and to environing structures may be determined by factors such as the hydrogen ion concentration through their effects upon the molecular arrangement at interfaces. While it is well known that the pH must be kept within relatively narrow limits for the maintenance and growth of most cells, it is surprising that so relatively little attention has been paid to the immediate effects of pH change upon the character of cell activity.

The studies reported below, carried out by means of direct observations on living cells and photographic records of their responses, have shown that changes in the pH of their environment can induce contraction and relaxation of cytoplasm, or activation and inhibition of its activity. These observations are in agreement with some of the earlier studies, particularly that of Lewis (10), and suggest that one of the mechanisms by which cells react to their surroundings may involve

in concentration of free CO_2 in solution, of the hydrated but un-ionized H_2CO_3, or of the dissociated ions, H^+ and HCO_3^-.

Since the concentration of dissolved CO_2 and of H_2CO_3 and its ionization products are proportional to the partial pressure of the CO_2 in the gas with which it is in equilibrium, one can write

$$\frac{(H^+) \ (HCO_3^-)}{(CO_2)} = K$$
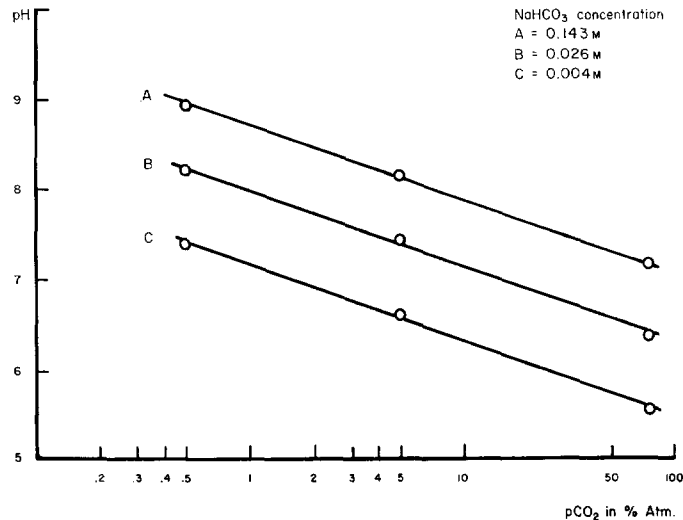
Expressed in logarithmic terms, this may be con-

FIGURE 1

Graph showing the relationship between pH and pCO_2 in the three media. Solution A, bicarbonate-enriched medium. Solution B, standard medium. Solution C, bicarbonate-deficient medium.

local pH changes occurring at their surfaces and possibly also within the cells.

MATERIALS AND METHODS

Test cells were obtained in single-cell suspension from freshly trypsinized chick embryo kidney, liver, and heart or from an established strain of human conjunctival cells released from attachment to culture flasks by Versene. After the cells had become spread on glass in perfusion chambers, they were photographed in phase contrast by cinemicrography at 6 frames per minute. Photography was not interrupted for changing the medium or perfusion gas.

The standard medium for culturing was Eagle's Basal Medium (5) supplemented with 10 per cent beef embryo serum. In the carbondioxide–bicarbonate buffer system of this medium the pH is generally altered with every change in CO_2 concentration. It, therefore, became of interest to determine whether any observed cell responses were the result of changes

verted into the well known Henderson-Hasselbach formulation

$$pH = \frac{pK + \log (HCO_3^-)}{(CO_2)}$$

This equation defines a relationship between pH, (CO_2) and (HCO_3^-) which permits varying any two in such a way as to keep the third constant. Thus, by altering the pCO_2 of the atmosphere and the concentration of $NaHCO_3$ in the buffer (which is almost completely dissociated), it should be possible to determine whether one variable alone is responsible for any observed cell reactions and, if so, which one of the three variables it is.

The partial pressure of CO_2 was varied by perfusing the cultures with gas. For this purpose, washed oxygen, nitrogen, and carbon-dioxide were obtained from commercial sources. When these gases were used alone, they are here referred to as "pure" to
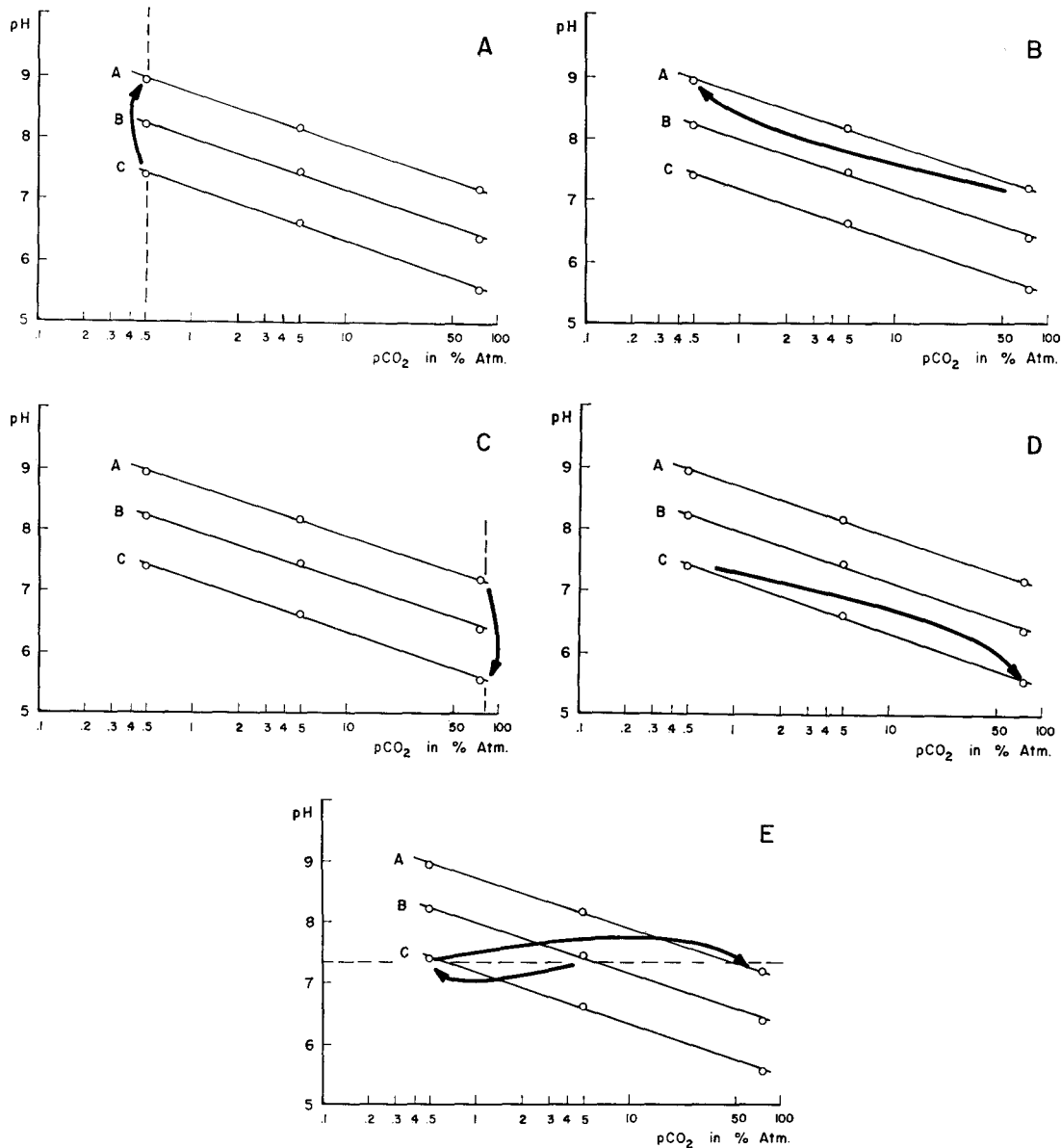
FIGURE 2

Diagrams of the procedures for varying the environmental conditions used in the different experiments. Raising (A) or lowering (C) the pH by changing the $NaHCO_3$ concentration in the medium without altering the pCO_2. Raising (B) or lowering (D) the pH by changing the pCO_2 without altering the buffer concentration. (E) Changing both the pCO_2 in the perfusing gas and the $NaHCO_3$ concentration of the medium without altering the pH.

distinguish them from mixtures of gases which were also used. The proportions in these mixtures were: 99.5 per cent air + 0.5 per cent CO_2, 95 per cent air + 5 per cent CO_2, and 22 per cent O_2 + 78 per cent CO_2. The concentration of O_2 in each of these mixtures was nearly equal, the differences being insignificant in the light of the experiments with O_2 mentioned below.

The concentration of $NaHCO_3$ was changed by replacing the medium of a culture with another of

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different composition. The standard medium (here designated as Solution B) was modified by altering the proportions of salts in the Eagle's component to make two other media: (1) a bicarbonate-enriched medium (Solution A) prepared by replacing the NaCl called for in Eagle's fluid with an osmotically equivalent amount of $NaHCO_3$, and (2) a bicarbonate-deficient medium (Solution C) made by reducing the amount of $NaHCO_3$ and restoring osmotic balance by an appropriate increase of NaCl.[1]

The combination of these variables used are shown in Fig. 1. The indicated pH values for each of the three solutions at different partial pressures of CO_2 were experimentally determined by a glass electrode pH meter. They are seen to fit closely the calculated values for each solution which are indicated by the straight lines A, B, and C.

In Fig. 2, A and B indicate the two methods used to elevate the pH from 7.3 to 8.9, and, similarly C and D show the methods used to reduce pH from 7.3 to 5.6. Diagram E (Fig. 2) shows the control conditions in which pCO_2 and $NaHCO_3$ concentration were changed without altering the pH.

Tests were also made with Media A, B, and C from which serum had been omitted, or in which the $CaCl_2$ had been replaced by an osmolar amount of NaCl. Tests were also carried out in media made with Tris (hydroxymethyl aminomethane) as a buffer instead of $NaHCO_3$, or without any added buffer, using HCl and NaOH to adjust pH.

RESULTS

Cells kept in cultures in Medium B and perfused with air + 5 per cent CO_2 would maintain a spread condition and exhibit normal uniform activity for many days. When, however, the perfusing gas was changed from that mixture to pure O_2, all cells began to contract and withdraw their processes within a few minutes (Fig. 3). Often points of attachment to glass or to other cells would persist for a time so that contracting cells remained moored by taut strands of protoplasm (cf. Fig. 4 B). Prolonging the O_2 perfusing for over an hour resulted in the detachment and withdrawal of most of these fibers. Attachments to the substratum generally broke before those to adjacent cells, thus allowing the freed cells to clump or ball together as shown in Fig. 3 B.

This contraction and detachment ceased if the perfusing gas was changed back to air + 5 per cent CO_2 before the cells had become injured. All cells then commenced to flatten on the glass and to form

[1] The calculation of osmotic equivalents of the different salts was based upon G values taken from International Critical Tables, 1929, 4, 254.

again a sheet (Fig. 3 C). Under these conditions, the return to a fully spread state generally required a longer time than did the contraction.

A similar response followed perfusion with pure N_2. In repeated observations, no distinction could be made between the appearances of cells during the 1st hour of perfusion with either O_2 or N_2. The similarity of these responses suggested that the observed effect was due not to the action of the gases with which the cultures were being perfused but to the lack of some component of the gas mixture which they replaced, presumably CO_2. The indifference of the cells to so wide a variation in concentration of O_2 in their environment appears to justify disregarding the difference of less than 1 per cent in O_2 content of the gas mixtures mentioned in the section on Materials and Methods.

When cultures were perfused with pure CO_2, cell activity became rapidly reduced. The cell margins, which had been in active movement, became quiescent and motion of cytoplasmic granules almost ceased so that by $\frac{1}{2}$ to 1 hour the cytoplasm of most cells was firmly gelated and appeared as if frozen or fixed. When CO_2 in the perfusion gas was again reduced to 5 per cent, cytoplasmic solation occurred and activity quickly returned. Even after CO_2 perfusion had been maintained for 2 hours, recovery of most cells was complete.

In the tests with Medium B just mentioned, changing the perfusion gas from pure O_2 to pure CO_2 represented an increase of pCO_2 from 0 to 100 per cent with an accompanying drop in pH from 8.3 to 6.1. To determine whether the change in pH or the change in CO_2 concentration was responsible for the observed cell behavior, Media A and C, with their different buffer concentrations, were used with the following results.

When the pH was raised from 7.3 to 8.9, either by changing the $NaHCO_3$ concentration of the medium (changing from Medium C to Medium A, Fig. 2 A) or by changing the partial pressure of CO_2 (using only Medium A, Fig. 2 B), a marked contraction and detachment of cells occurred which was similar to that observed in pure O_2. This was completely reversible after exposures of $\frac{1}{2}$ hour or less (Fig. 4). Among the cell types tested, a difference was observed in their threshold of response to an increase of pH. Epithelial cells were the first to show an effect and reacted more strongly to the same level of pH than did endothelial or stroma cells from the same organ. Of the

latter two types, the stroma cells were the least affected.

Raising and lowering the pH in steps showed that a response was detectable after a change as small as 0.2 of a pH unit. For human conjunctival cells, activity was progressively increased when the pH was raised between 7.4 and 8. This included speeding up of activity of the margin, pinocytosis, and the motion of granular cell inclusions. Between pH 8 and 8.3, contraction and detachment of cells occurred.

Long exposure to high pH resulted in failure of some cells to recover. In most cases, alkali treatment was discontinued when the cells had rounded and started to detach. At pH above 8.5 this required only 10 to 15 minutes; however, cells kept at pH 8.2 for 45 minutes recovered completely.

When the pH was reduced from 7.3 to 5.6, either by changing the bicarbonate concentration of the medium (Fig. 2 C) or the pCO_2 of the perfusing gas (Fig. 2 D), an immobilization of all cell components occurred. Evidence of this effect appeared first at the periphery as a quieting of activity of the cell margin. Cell processes ceased their movements or were withdrawn, although the cells remained spread. This was followed by essentially complete standstill of all granular inclusions, mitochondria, Golgi zone, and the nucleus itself. The latter eventually lost its normal homogeneous hyaline appearance and became finely granular (Fig. 5). As treatment was prolonged, these granules appeared to aggregate into diffuse clumps and also to become adsorbed to the nuclear membrane, making it stand out in strong contrast. The nucleoli also became more condensed, as indicated by their apparent shrinkage and a change in their optical density. The latter frequently resulted in a reversal of their phase contrast from dark to light (Fig. 5). Division of those cells which had already begun the process of mitosis was checked by low pH, and no new mitoses appeared for as long as 18 hours. Rhythmically beating embryonic heart cells ceased their beating in the low pH medium.

When the pH was returned to 7.4, the recovery steps occurred in the same order. Peripheral activity began first, followed by the return of motion of cytoplasmic components. Arrested mitotic activity and beating of heart cells were resumed.

Most cells of cultures kept at pH 6.5 for 1 hour recovered without apparent damage. After prolonged exposure or treatment with stronger acid,

evidence of cell damage was seen in the appearance of vacuoles in the cytoplasm and the development of blebs or bubbling when the cells were returned to neutral medium. If, however, quiescent or "frozen" cells were maintained at low pH, they remained unchanged in appearance until suddenly all would simultaneously explode. In two instances, this occurred at pH 6.3 after 16 hours and at pH 6 after 5 hours and 20 minutes. The reason for this occurrence was not apparent.

The cell responses just described were not caused by changes in the concentration of the bicarbonate or of the CO_2. This became evident from the experiment in which these variables were changed to the same degree as before, but in such a way as to leave the pH unaltered (Fig. 2 E). In this test, the cells retained the same appearance and activity rate in all three solutions and during perfusion with 0.5, 5, and 78 per cent CO_2. It is apparent that when the concentrations of HCO_3 and CO_2 were raised or lowered simultaneously the level of undissociated H_2CO_3 in the medium was also proportionately changed. Thus, of the four factors varied, only changes in pH were effective in producing the responses observed.

When the pH was varied as in the experiments described above, but with the cultures in medium which contained no calcium or magnesium except that which was contributed by the added serum, the cell responses were still the same as in the complete medium. The fact that the characteristic behavior of cells was not affected by a reduction of approximately 90 per cent in the calcium and magnesium content of their environment suggested that these ions are not importantly involved in the pH reaction. Likewise, cell reaction to pH change was shown to be independent of the presence of serum. Despite the important role played by serum in the attachment and spreading of cells on glass, as described in a previous publication (19), the characteristic reactions to pH change were noted when serum was completely omitted from the medium.

To prove that the observed pH effect was not peculiar to behavior in the buffering system which was used in the experiments described above, tests were conducted without $NaHCO_3$. The same responses were obtained when Tris was used as the buffer or when no buffer was present and pH was adjusted with HCl and NaOH.

It was noted that a reversible gelation of cytoplasm and inactivation of cells similar to that
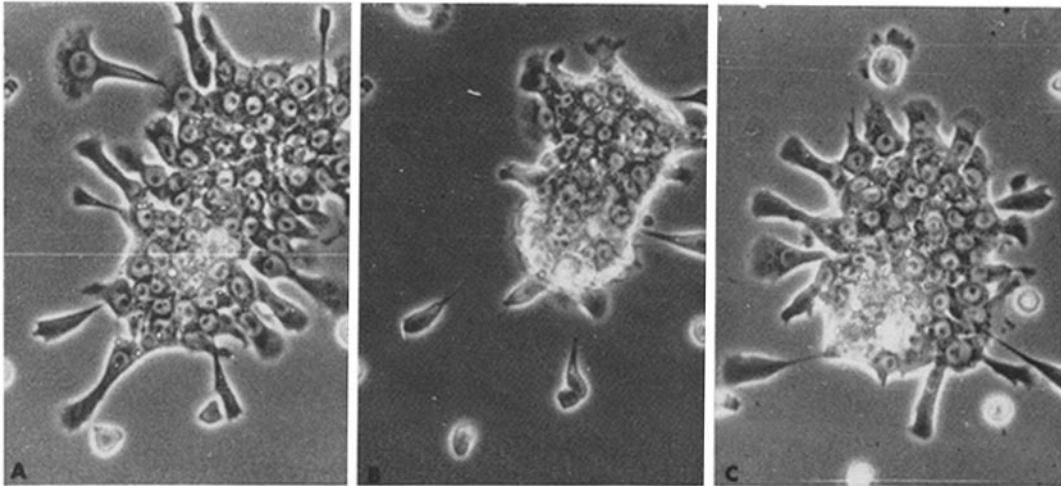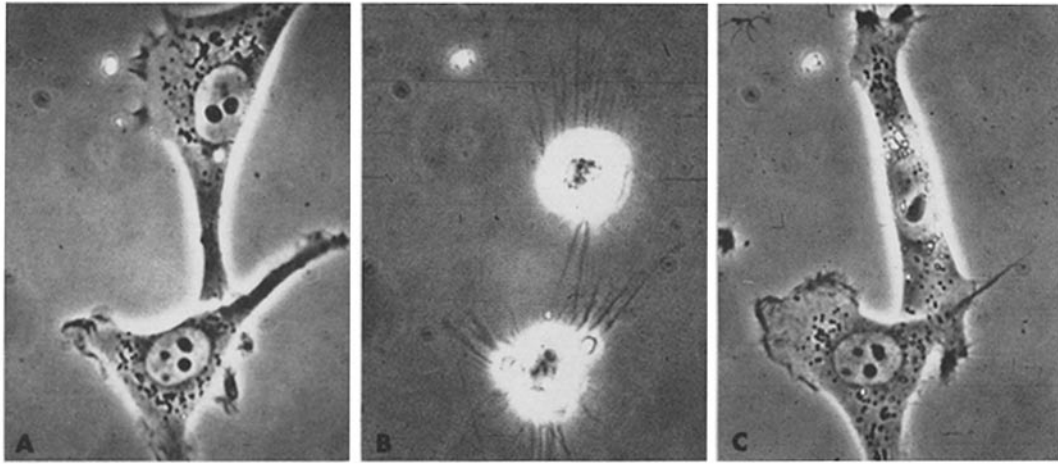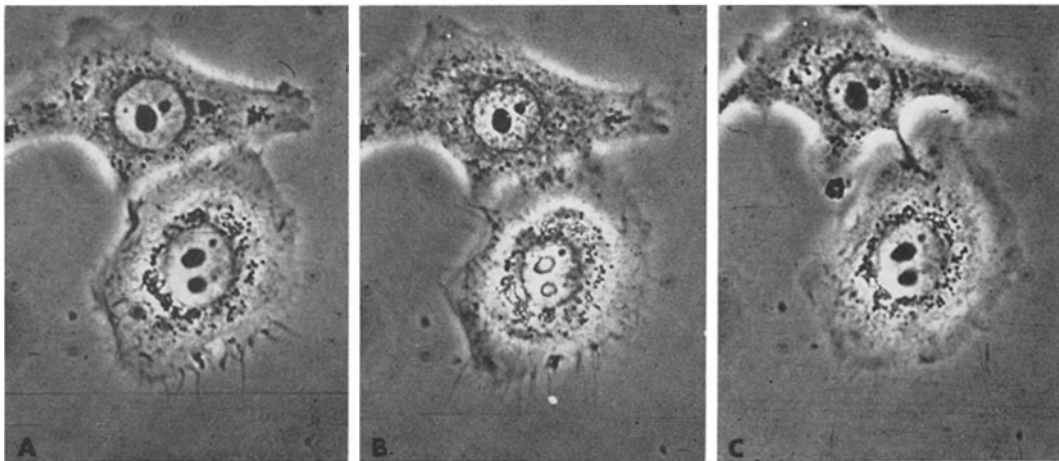
FIGURE 3

FIGURE 4

FIGURE 5

produced by low pH was obtained when cells were perfused with medium of the same pH but made hypertonic by the addition of 4 per cent dextrose. On the assumption that the low pH effect might be due to loss of water from the cell as a result of cell membrane changes, cultures which had been immobilized by pH 5.8 were then perfused with medium diluted with an equal volume of distilled water but still at a pH of 5.8. These cells promptly regained their activity. Apparently, the effect of acid medium could be corrected by a hypotonic environment. The reciprocal reversal, however, did not occur when cells which had been induced to contract by high pH were placed in hypertonic medium. Such cells remained contracted even when the osmotic pressure in the medium was doubled.

DISCUSSION

These experiments offer little insight into the mechanism by which change in environmental pH may elicit in cells the characteristic responses here described. Studies on intracellular pH (see reviews by Caldwell, 3 and by Hill, 8) have shown that many cells may be highly resistant to changes of their internal pH, due, presumably, to the impermeability to ions of their surface membranes and to effective buffers in their protoplasm. However, particular acidic and basic substances, notably carbon dioxide, were found to be effective in changing intracellular pH. This fact has been interpreted to mean that the un-ionized molecules may penetrate into cells more easily than electrically charged ions.

The work here reported has shown that it was the concentration in the medium of hydrogen ions, rather than of carbon dioxide or carbonic acid molecules, which was responsible for the cell reactions. Whether these ions exerted their effect on the cells by penetrating their membranes and changing the pH of their cytoplasm is not certain. Attempts to answer this question were made by observing the color of neutral-red indicator dye, which previously had been taken up within living cells, when the cells were then placed in different test environments. The attempts failed to show any evidence of internal pH change under the different test conditions of this experiment. This result and the reported impermeability of cell membranes to hydrogen ions suggest that the effect of environment upon the cells which was responsible for their reactions may have been something other than the alteration of their internal pH. This effective change might be the modification of the surface of the cells, involving, for example, changes in the distribution of charged groups on the membrane proteins, modifications of the rates of activity of local enzymes or variations of the permeability of the cell membranes to substances in the medium or within the cells.

In these experiments, cells responded to changes of pH in the total environment. Recently, however, experiments in our laboratory (16) have

FIGURE 3

Enlarged frames from a motion picture of chick embryo liver cells spread in Standard Medium. A, perfused for 24 hours with air + 5 per cent CO_2. B, same cells after perfusing with pure O_2 for ½ hour. C, same culture ½ hour after returning to air + 5 per cent CO_2.

FIGURE 4

Enlarged frames from a motion picture of human conjunctival cells in Medium A and treated as in Figure 2 B. A, after perfusing for 4 hours with 22 per cent O_2 + 78 per cent CO_2 (pH 7.3). B, same cells 10 minutes after changing perfusion gas to 95.5 per cent O_2 + 0.5 per cent CO_2 (pH 8.9). C, same cells 20 minutes after returning to 22 per cent O_2 + 78 per cent CO_2.

FIGURE 5

Enlarged frames from a motion picture of human conjunctival cells in Medium C and treated as in Fig. 2 D. A, after 4 hours' perfusion with 95 per cent air + 0.5 per cent CO_2 (pH 7.3). B, same cells 40 minutes after changing perfusion gas to 22 per cent O_2 + 78 per cent CO_2 (pH 5.6). C, same cells after return to 95.5 per cent air + 0.5 per cent CO_2.

indicated that a cell will change its polarity and the direction of its locomotion in response to a micro-stream of fluid applied so as to affect the pH of only a portion of its surface. This is evidence that localized pH differences may be sufficient to strongly influence, or modify, the activities of cells.

These responses of isolated cells to experimental changes in pH of their environment suggest a possible role for pH in determining the interactions between cells when they occur as members of a population, either *in vitro* or *in situ*. In this case, the cells themselves would be the cause of the pH change. It might, however, be questioned whether, in tissue culture or in their normal environment in the organism, cells would encounter pH differences of adequate magnitude and sufficient localization to be effective in influencing their activities. Studies by Danielli (4) and by McLaren (13) have emphasized that the pH of the bulk phase may be quite different from that on a cell membrane or at a colloidal interface. Danielli estimates that these may differ by as much as 2 pH units. It thus seems feasible to assume that, even *in vivo*, variations of pH may exist in the microenvironment of local areas on a cell membrane which would be adequate to induce gelation or contraction of the adjacent cytoplasm and thus bring about asymmetrical cell change.

Seifriz (17) found that carbon dioxide, like other anesthetics, causes gelatinization and cessation of movements in protoplasm. That carbon dioxide may exert a depressing or narcotic effect on nerve cells, in particular, has frequently been mentioned in medical literature. The effect has sometimes been attributed to anoxia since the proportion of oxygen was often reduced in its administration. However important an adequate oxygen tension may be for prolonged maintenance of cells in a healthy, active condition, it is quite clear from the experiments described that the inhibition of cell activity and cytoplasmic congelation resulting from increased carbon dioxide tension was not due to lack of oxygen. This effect was, furthermore, not produced by a direct action upon the cells of CO_2 as a molecular species, but rather by its contribution of hydrogen ions through the dissociation of its hydrated form. In fact, the effectiveness of carbon dioxide in altering the pH of biological systems and its prevalence as a product of metabolism would give it an important

indirect role in any regulation of cell movements by the pH effect.

Examples of cell behavior *in vitro* can be singled out which might be interpreted as showing the influence of pH on cell activity. One such is the greater surface activity of cells, or parts of cells, which are exposed to free exchange with a buffered liquid medium as compared to cells, or parts of cells, which are applied to the glass substratum or are in contact with other cells. This is particularly noticeable in islands of epithelial cells spread on glass in a liquid medium where invariably the cells within the island are less active than those at the margins of the group, and the latter are always less active where they are in contact with other cells than along their free edges. The effect might be attributed to carbon dioxide and other products of metabolism or secretion of cells which accumulate between apposed surfaces and result in a lowered pH with its inhibiting action.

Other examples of such a regulation of cell activity may be illustrated by the "snapping" apart of chick embryo heart cells in culture after they have come together (22) or the "contact inhibition" of stroma cells described by Abercrombie (1). These occurrences would result from a local gelation, or narcotization, due to a local lowering of pH between those portions of the two cells which had made contact. The continued activity of the remaining cell surface, being exposed to an environment of higher pH, would then carry the cells apart again. After separation of the cells, the paralyzed portions may then recover their former pH and their activity.

The mutual inhibition of like epithelial cells in development and tissue repair, which has been discussed by Weiss (21), involves an aspect of specific recognition by the cells which obviously cannot be accounted for by so simple a mechanism as the pH effect. If, however, specific cell interactions require the combined effort of a primary selective recognition which then triggers secondary non-specific implementing action (23), the pH effect might then operate as the non-specific portion of this two-step process.

In the examples just cited, there is no evidence that pH differences are responsible for the behavior described. They are mentioned merely to show that certain activity patterns of cells *in vitro* and *in vivo* resemble the responses of isolated cells to the experimental changes of pH described in

these studies. It seems clear that cell activity is responsive to variations in pH such as may occur normally in the cell's environment. Whether, in fact, local pH changes are instrumental in regulating the activity of cells observed in cultures and in their normal surroundings remains to be determined.

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